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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Preparation and Use of Gene Banks of Human Antibodies ("Human-Antibody Libraries")

(72) Little, Melvyn - Germany (Federal Republic of) ;
Breitling, Frank B. - Germany (Federal Republic of) ;
Seehaus, Thomas - Germany (Federal Republic of) ;
Dübel, Stefan - Germany (Federal Republic of) ;
Klewinghaus, Iris - Germany (Federal Republic of) ;

(73) Behringwerke Aktiengesellschaft - Germany (Federal Republic of) ;

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BEHRINGWERKE ARTIENGESELLSCHAFT HOE 90/B 005J - Ma 833
Dr. Lp/rd

Abstract of the disclosure

Preparation and use of gene banks of human antibodies ("human-antibody libraries")

The invention relates to the preparation and use of gene banks of human antibodies (Ab). Starting from a mixture of human B-lymphocytes, their mRNA is translated into the cDNA using oligo-dT primers. Subsequently, an amplification of the Ab-specific cDNA by means of polymerase chain reaction (PCR) takes place using suitable oligonucleotide primer sequences. Expression of this amplified Ab-specific cDNA in a bacterial expression vector, e.g. the vector pFMT described below, in E. coli thus makes available a human-antibody library with a comprehensive repertoire for screening selected antigens in vitro.

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Description

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The invention relates to the preparation and use of gene banks of human antibodies (Ab). Starting from a mixture of human B-lymphocytes, their mRNA is translated into cDNA using oligo-dT primers. Subsequently, an amplification of the Ab-specific cDNA by means of polymerase chain reaction (PCR) takes place using suitable oligonucleotide primer sequences. Expression of this amplified Ab-specific cDNA in a bacterial expression vector, e.g. the vector pFMT described below, in E. coli thus makes available a human-antibody library with a comprehensive repertoire for screening selected antigens in vitro.

The human or mammalian immune system comprises an estimated number of between 10^5 and 10^6 different antibodies. This number of antibodies seems to be sufficient to cause an immune reaction of the body both against all naturally occurring antigens and against artificial antigens. If it is furthermore taken into account that often several antibodies react with the same antigen, the repertoire of antibodies that are really different would be rather in the region from 10^4 to 10^7.

Up to now specific antibodies have always been obtained starting from an immunization with the particular antigen, for example injection of the antigen into the body or in vitro incubation of spleen cells with this antigen. In the case of polyclonal antibodies, the immunoglobulins can then be isolated from the serum and the specific antibodies can be isolated therefrom, e.g. by absorption methods. Monoclonal antibodies are isolated from the cell

supernatants or from the cell lysate of spleen tumor cells (hybridoma cells) which have been fused with individual B lymphocytes and cloned. The abovementioned methods are unsuitable in particular for the preparation of specific human antibodies or human monoclonal antibodies.

The present invention therefore haa the object of developing a generally usable process for generating specific human monoclonal antibodies (huMAbs) or parts of antibodies, which contain the antigen binding site.

It has been found that the desired huMAbs or parts thereof which contain the variable, antigen binding domain can be isolated from gene banks of human immunoglobulins. First of all, atarting from a mixture of nonactivated human B-lymphocytes, their mRNA was isolated and translated into cDNA with the aid of oligo-dT primers. A specific amplification of the population of antibody cDNAs within the resulting cDNA pool was achieved by using PCR. For thia purpose certain oligonucleotide primers which are homologous to conserved sequences at both ends of the antibody cDNA were used (see below and examples). The design of the primer for the reverse reaction for the synthesis of the noncoding strand of the DNA of the heavy chains is based on IgM sequences (subclass III, since this comprises most of the IgM sequences). IgM molecules occur more often in nonactivated B-lymphocytes than all other immunoglobulin classes. In contrast, IgG sequences predominate in activated B-lymphocytes whose repertoire of different antibodies is very much smaller. An IgG library would additionally entail the danger of one or a few particularly strongly expressed IgGs dominating the library.

Up to 30 amplification cycles were, advantageously, carried out. The oligonucleotide primers contain suitable restriction sites for inserting the amplified DNA e.g. into the antibody expression plasmid pFMT (aee below).

This expression plasmid makes possible the expression of antibody cDNA and subsequent secretion of the expression products in bacteria (E.coli). The antibody operon of the plasmid contains the sequences of the variable parts of both the heavy and light chain of an antibody. Suitable leader sequences from the amino terminal part of a bacterial protein makes secretion of the antibody parts possible. The leader sequences are cleaved off by a bacterial enzyme during the secretion. During the secretion of the antibody cDNA products, the light and heavy chains of the antibody (with or without an adjacent constant domain) become associated. This results in the formation of an antibody or antibody fragment which, in either case, contains a functional antigen binding site. Similar constructs for individual antibodies have also been described by other authors (Better et al. (1988), Science 240, 1041, and Skerras & Plückthun (1988), Science 240, 1038).

It is true that the amplification of DNA coding for the variable parts of antibodies has been described by other authors (Orlandi et al. (1989), Proc. Natl. Acad. Sci. 86, 3833; Sastry et al., (1989) Proc. Natl. Acad. Sci. 86, 5728; Ward et al. (1989), Nature 341, 544); Huse et al. (1989), Science 246, 275). In this case however, the mRNA which, inter alia, also codes for antibodies was isolated from hybridoma cells or spleen lymphocytes after treatment with a certain antigen. For this reason primer sequences which are based only on IgG sequences were also used there. This is, of course, an advantage if as many antibody DNA clones as possible which are derived from activated lymphocytes are sought. With primers from IgG sequences, the chances of finding clones which contain DNA coding for antibodies against the injected antigen are much higher. It has to be added that in the foregoing papers murine and therefore nonhuman antibody DNA was synthesized and, additionally with exclusion of regions of the lambda chain, amplified.

The present invention, in contrast, uses primer sequences which are homologous to the sequences in the constant domains of IgM cDNA. This is the best way of implementing the invention, i.e. making available a very large choice of antibodies, namely the whole antibody repertoire, in the form of a library. The expression in, preferably, E. coli then results in the desired human-antibody library in which the desired human antibodies or antibody parts are found by means of screening bacterial clones using the selected antigen.

Oligonucleotide primers suitable for amplification are compiled in Tab. 1. The positions of the abovementioned primers on the μ, kappa and lambda chains are shown in the form of a diagram in Tab. 2. The molecular biological constructions of, amongst others, the expression vector, i.e. the antibody expression plasmid pFMT, are described in detail in the examples below.

The invention therefore relates to human-antibody libraries, prepared by transcription of the mRNA from nonactivated (peripheral) human B-lymphocytes by means of oligo-dT primers, subsequent amplification by PCR using primers containing sequences which are homologous to conserved regions of the IgM cDNA, and subsequent incorporation into suitable expression plasmids for the expression in microorganisms, preferably in the expression vector pFMT for the expression in E. coli. In a preferred embodiment an additional sequence is incorporated which codes for a marker peptide, e.g. a TAG sequence so that the expression products can be detected in a simple manner using established monoclonal antibodies against the marker peptide (Wehland et al., (1984), EMBO J. 3, 1295).

The invention also relates to the use of abovementioned human-antibody libraries for isolating desired human antibodies or parts of antibodies containing a functional antigen binding site by screening using selected

antigene, and to a process for isolating the said human antibodies or their antigen-binding parts, and also to a process for preparing the said human-antibody libraries.

The invention also relates to expression vectors having the properties of the antibody expression plasmid pFMT.

The examples below further illustrate the invention without restricting it. Finally, the invention is also contained in the patent claims.

Examples:

Example 1: Preparation of an antibody expreseion vector

The plasmid pKK233-2 (Amann and Brosius, (1985) Gene 40, 183 and Straus and Gilbert, (1985) Proc. Natl. Acad. Sci. 82, 2014) was chosen as base vector for the construction of the antibody expression vector (Fig. 1).

Before the incorporation of the antibody operon, the plasmid was cut with SalI and BamHI, the ends were filled in with Klenow polymerase and ligated. By doing so, the two restriction sites and the DNA between them were removed. Additionally, the plasmid was cleaved with HindIII, the ends were filled in with Klenow polymerase and ligated using BamHI linkers. By this procedure, the HindIII restriction site was removed and a BamHI site inserted. The antibody DNA was inserted into this modified plasmid. A diagrammatic route for construction of the antibody operon which codes for a bicistronic antibody mRNA is shown in Tab. 3. In order to make possible the secretion of the antibody, the leader sequence of the bacterial enzyme pectate lyase was used. The leader sequence of this enzyme has already been used for the expression and secretion of a chimeric murine/human antibody (Fab fragment, Better et al., loc. cit.), and of the variable part of a "humanized" antibody (Ward et al.,

loc. cit.; Huse et al., loc. cit.). DNA for the first leader sequence (P_1 upstream of the heavy chain), and the sequence for a second ribosome binding site (RBS) and a second leader sequence (P_2 upstream of the light chain) were synthesized from several oligonucleotides (Tab. 4).

Antibody cDNAs which code for the variable regions of the heavy and light chains of a human antibody (HuVhlys or HuVllys; Riechmann et al., (1988) J. Mol. Biol. 203, 825) were obtained from Dr. G. Winter (Cambridge, UK). The restriction sites HindIII (HuVhlys) and EcoRV (HuVllys) were introduced to make possible the insertion of the antibody cDNA into the expression vector. Further restriction sites for BanII (HuVhlys) and BstEII or KpnI (HuVllys) were introduced to exchange hypervariable regions en bloc. At the end of the HuVhlys cDNA sequence a stop signal was incorporated. A BanII site in the light chain was removed. These alterations were carried out by means of site directed mutagenesis in the bacteriophage M13mp18 (Zoller and Smith, Meth. Enzymol. 100, 468-500). The sequence of the completed antibody DNA is shown in Tab. 5.

For the insertion of the leader sequence P_1 (Tab. 4) the modified plasmid pKK233-2 was digested using the restriction enzymes NcoI and PstI, and P_1 was inserted in between these sites (pKK233-2-P_1). Further cloning steps, apart from the last step, were carried out using the plasmid pUC18. The reason is that the presence of individual parts of the antibody operon in the expression vector adversely influences the growth of the bacterial host.

Before the cloning in pUC18, its BamHI restriction site had to be removed. After digesting with BamHI, the single-stranded ends were filled in using the Klenow fragment and were religated. This modified plasmid was then digested using PstI and HindIII, and P_2 plus RBS was ligated in between the restriction sites (pUC18-P_2).

During this process, the original HindIII restriction site of the plasmid disappears and a new HindIII restriction site is incorporated. pUC18-P_2 was then digested using PstI and HindIII, and the DNA of the heavy chain (PstI-HindIII insert from M13) was ligated into these two sites (pUC18-HP_2). This plasmid was then digested using EcoRV and BamHI, and the DNA of the light chain (EcoRV-BamHI insert from M13) was ligated in (pUC18-HP_2L).

In a preferred embodiment a Tag sequence was ligated into the new HindIII cleavage site (Tab. 4). The Tag sequence codes for the peptide Glu-Glu-Gly-Glu-Glu-Phe and is recognized by the monoclonal antibody YL 1/2 (Wehland et al. (1984) EMBO J. 3, 1295). The resulting plasmid is pUC-HTP_2L.

For the insertion of HP_2L or HTP_2L into the expression vector, pUC18-HP_2L or pUC-HTP_2L, respectively, were cut using PstI and BamHI, and the relevant restriction fragment was ligated into these two restriction sites in the modified plasmid pKK233-2-P_1, in each case. A diagrammatic representation of the completed expression vector pFMT is shown in Tab. 6.

Example 2: Isolation of RNA from human B-lymphocytes

To enrich peripheral B-cells from human blood, this was diluted 1:1 with PBS (phosphate buffered saline) and centrifuged on a cushion of Ficoll® (Pharmacia) (1,077 kg/l). The cells of the interphase were washed twice with PBS and were incubated at 37°C for one hour on a plastic surface (culture bottle) in RPMI medium containing 10% fetal calf serum. The adherent cells (monocytes and macrophages) adhere to the culture vessel and it was possible in this way to remove them from the preparation. The nonadherent cells were collected by centrifugation and homogenized in 4.4 M guanidinium isothiocyanate, 5% mercaptoethanol and 2% lauroylsarcosine. The homogenate was then centrifuged on

a cushion of 5.7 M CsCl at 125,000 g for 18 hours. The sedimented RNA was dissolved in double-distilled H_2O and precipitated at -20°C overnight using 70% ethanol and 1/20 volume of 8M LiCl.

In order to obtain an even larger variety of antibodies of different specificities, RNA preparations of, in each case, 500 ml of the blood from 20 different people were mixed.

Example 3: Amplification of antibody DNA

The mRNA was purified on oligo-dT-Sepharose (kit supplied by Pharmacia) and translated into the cDNA by means of reverse transcriptase (kit supplied by Amersham) and oligo-dT primer. The products were used directly in the polymerase chain reaction (PCR). PCR primers and hybridization sites are shown in Tab. 1 and 2. Two different expression banks were produced by combining the μ-DNA obtained with either kappa- or lambda- DNA in the vector pFMT. The use of different primers for the synthesis of the noncoding strands in the polymerase chain reaction makes possible the preparation of two different antibody types which contain, in one case, only the variable domain and, in the other case, additionally a constant domain (similar to the Fab fragment of an antibody). For the PCR, 4 μl of a cDNA synthesis were reacted with 0.2 nmol of each of the two primers in a volume of 50 μl. The reaction mixture contained 100 mM KCl, 0.1% gelatin and 2.5 U of Taq polymerase. After 30 polymerization cycles comprising 1 min at 95°C, 2 min at 55°C and 2 min at 72°C, the DNA was precipitated using ethanol.

Example 4: Insertion of the antibody DNA into the expression plasmid

The precipitated DNA was taken up in application buffer for agarose gel (0.1% bromophenol blue, 7% Ficoll® [Pharmacia]) and fractionated in TBE buffer (45 mM

tris/borate pH 8.0, 10 mM EDTA) on 2% agaroae at 10V/cm. The antibody DNA synthesized was identified by meant of its molecular weight and eluted from the gel. It w. precipitated using ethanol and then taken up in buff- for the particular reatriction enzymes and cut with the appropriate (cf. Tab. 1 and 2) restriction enzymes (Boehringer Mannheim). After precipitation in ethanol, it was ligated into the vector pFMT cut in the same way, as is ahown in the form of a diagram in Tab. 7.

Example 5: Expression and screening antibodies in E. coli

Competent E. coli are transfected with pFMT plasmids containing the inserted antibody-DNA library, grown on agarose plates and then incubated using nitrocellulose filters coated with the desired antigen. After removing non-specifically bound antibodies, the active clones are identified with a labeled antibody against the human immunoglobulins secreted from E. coli. In the preferred embodiment, the monoclonal antibody YL 1/2 which is directed against the Tag sequence is used to identify the desired clones.

Legend for Fig. 1:

Restriction map of the expreaaion vector pKK233-2 (Amann and Broaius, loc. cit.).

Ptrc denotes hybrid tryptophan lac promoter

RBS denotes riboaome binding aite

rrnB denotes riboaomal RNA B (5S RNA)

5S denotes gene for 5S RNA (contains rrnB)

Before cloning antibody DNA in the expreasion vector, the following alterations were carried out:

1) The SalI and EcoRI restriction aites were removed together with the DNA between them.

2) The HindIII restriction aite was converted into a BamHI restriction site.

TAB. 1

Oligonucleotide primers for the amplification of cDNA uaing the polymerase chain reaction.

1. Oligonucleotide primers for the forward PCR

D. μ chain

GAGGTGCAG<u>CTGCAG</u>GAGTCTGGGGGAGGCTT (PstI)

E. kappa-chain

TGTCTGCATCTGT(A/G)GGAGACAG<u>GGTCACC</u>ATCA(A/C)TTG (BstEII)

F. λ chain

CCTCAG(C/T)GTCTGGG(A/T)CCCCAGGACAGAG<u>GGTGACC</u>ATCTCCTGC (BstEII)

2. Oligonucleotide primers for the backward PCR (variable domain plus adjacent conatant domain)

A1. μ chain (without Tag sequences)

GGGTGGGACGAAG<u>AAGCTT</u>ACTTAGGGAGGCAGCTCAGCAATCAC (HindIII)

A2. μ chain (with Tag aequences)

GGGTGGGACGAAGAAGCT<u>AAGCTT</u>GGGAGGCAGCTCAGCAATCAC (HindIII)

B. κ chain

GGCACTTC<u>GGATCC</u>TAACACTCTCCCCTGTTGAAGCTCTTTGTGACGGGCGA GCTCAGGCC (BamHI)

C. λ chain

GTGAGGG(A/T)TG<u>GGATCC</u>TATGAACATTCTGTAGGGGCCACTGT (BamHI)

3. Oligonucleotide primers for the backward PCR (variable domain)

G1. μ chain (without Tag sequences)

CACAGGAGACGAGGGGGAA<u>AAGCTT</u>TGGGGCTTATGCACTCCC (HindIII)

G2. μ chain (with Tag sequences)

CACAGGAGACGAGGGGGAA<u>AAGCTT</u>TGGGGCGGATGCACTCCC (HindIII)

H. κ chain

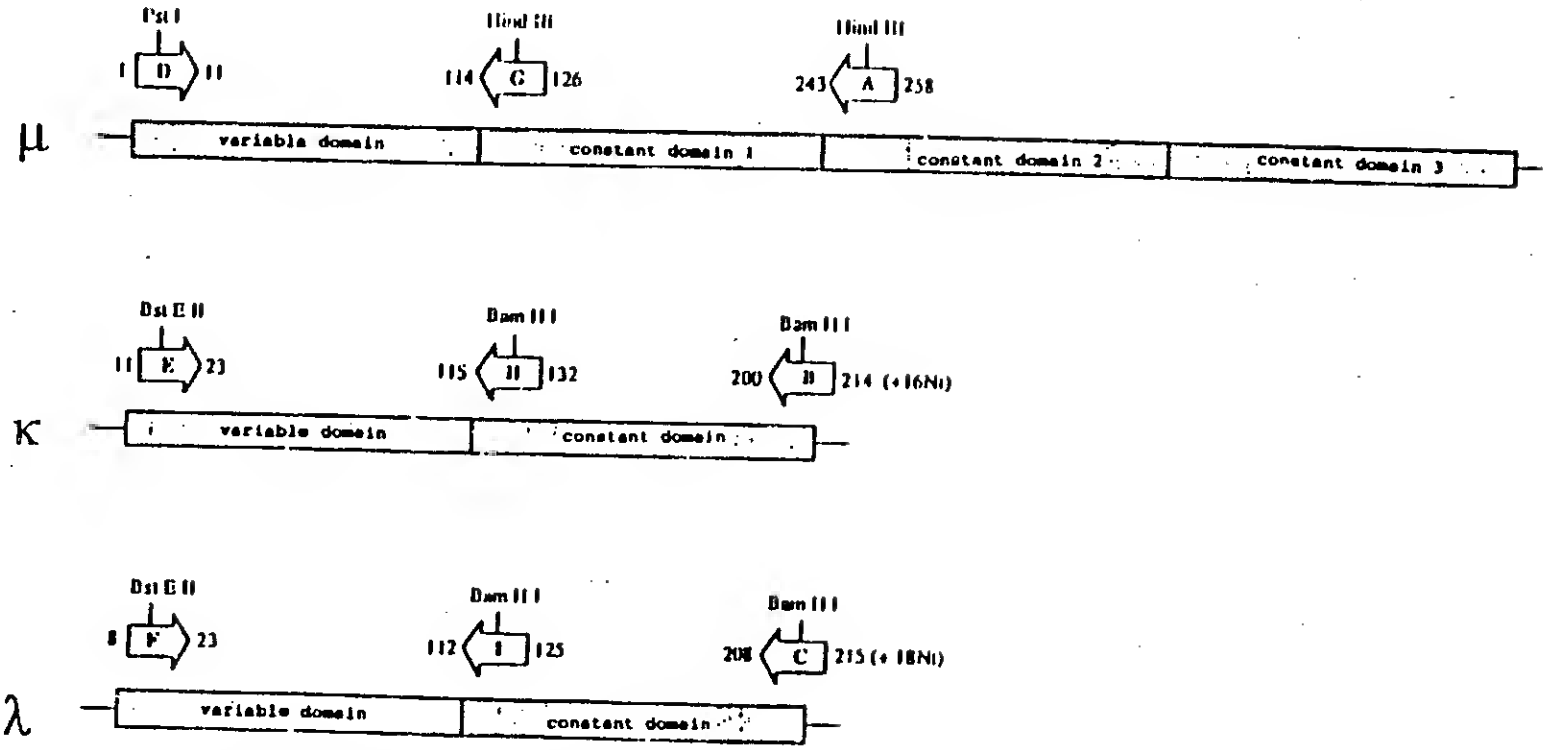
AACAGAGGC<u>GGATCC</u>TCATTTCAACTGCTCATCAGATGGCGGGAAGATGAA GAC (BamHI)

I. λ chain

AGCTCCTCAGAGGA(C/G)GGCG<u>GGATCC</u>GAGTGACCTAGGGG (BamHI)

TAB. 2

Positions of the primers for the polymerase chain reaction (PCR) of antibody DNA.



The individual experiences are listed in Tab. 1. The numbers indicate the position of the amino acids from which the particular primers are derived.

TAB. 3

CONSTRUCTION OF THE VECTOR pFMT FOR THE EXPRESSION AND SECRETION OF ANTIBODIES IN BACTERIA

ONA OF THE VARIABLE DOMAIN OF A HUMAN LYSOZYME ANTIBODY

↓

INTRODUCTION OF RESTRICTION SITES BY SITE DIRECTED MUTAGENESIS

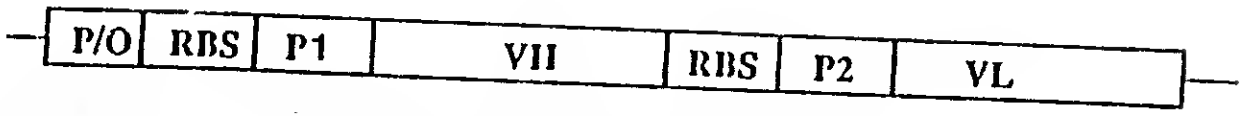
↓

SYNTHESIS OF THE LEADER SEQUENCE OF PECTATE LYASE AND OF THE RIBOSOME BINDING SITE

↓

LIGATION INTO BACTERIAL EXPRESSION PLASMIDS

↓



P/O: promoter/operator, RBS: ribosome binding site, P2: leader sequence of pectate lyase, VH: variable domain of the heavy chain, VL: variable domain of the light chain

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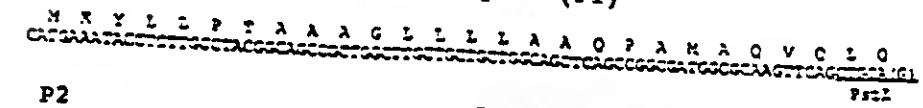
Tab. 4 Sequences of the leader eequences P1 and P2 in the antibody operon, and of the Tag sequences

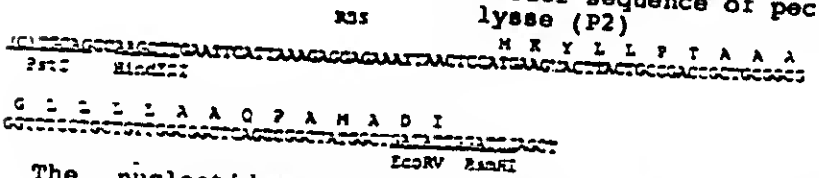
P1

Leader sequence of pectate lyase (P1)



P2

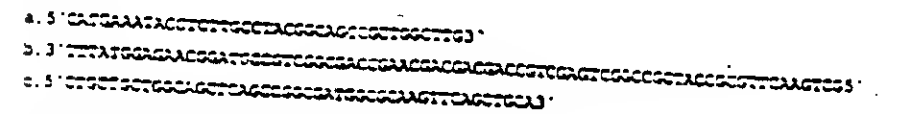
Lesder sequence of pectate lysse (P2)



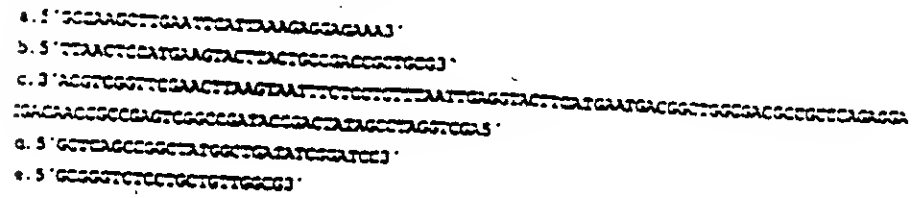
The nucleotides in parenthesis are the sdjacent nucleotides of the plasmid.

The leader sequences were synthesized by hybridizstion of the following oligonucleotides

P1



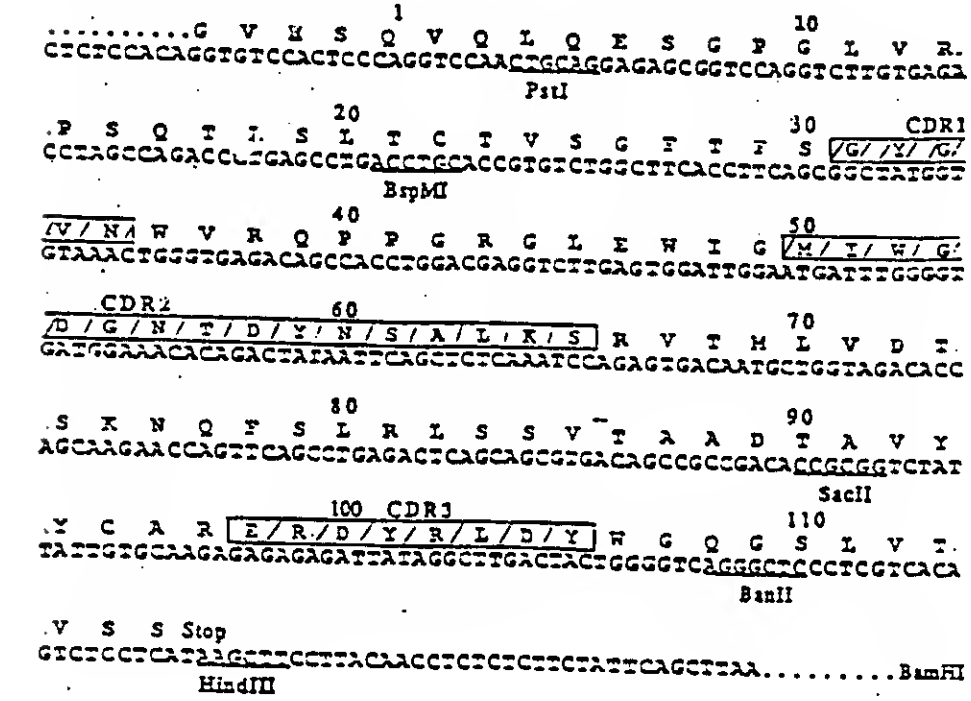
P2



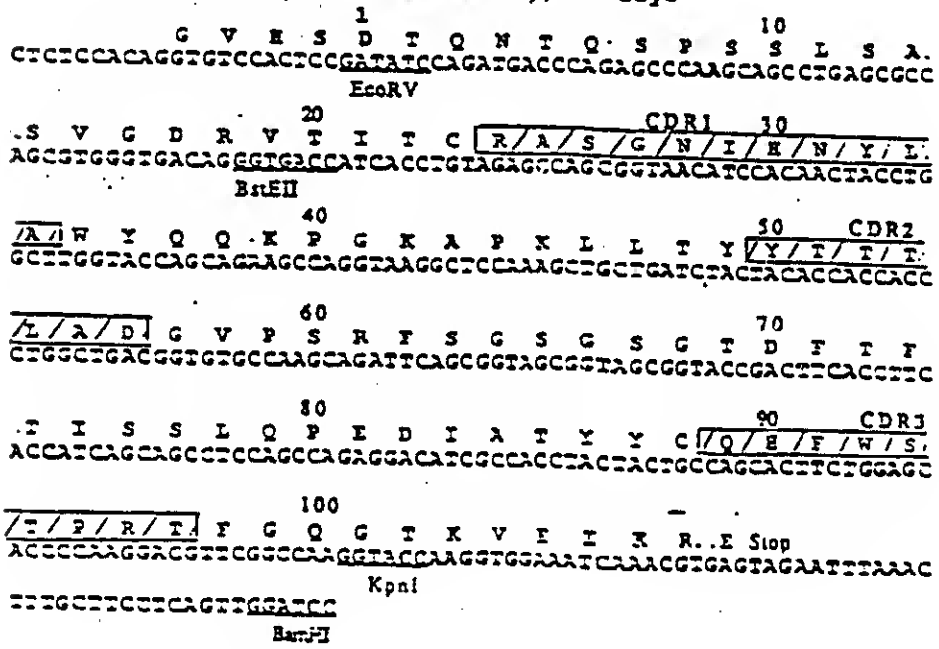
The Tag sequences were synthesized by the hybridization of the following sequences:

TAB. 5 Nucleotide sequences of antibody DNA

a) Heavy chain (variable domain), HuVhlys HindIII.....

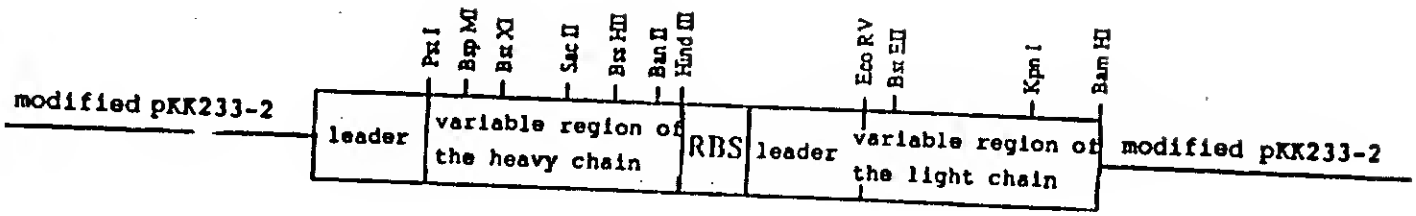


b) Light chain (variable domain), HuVllys



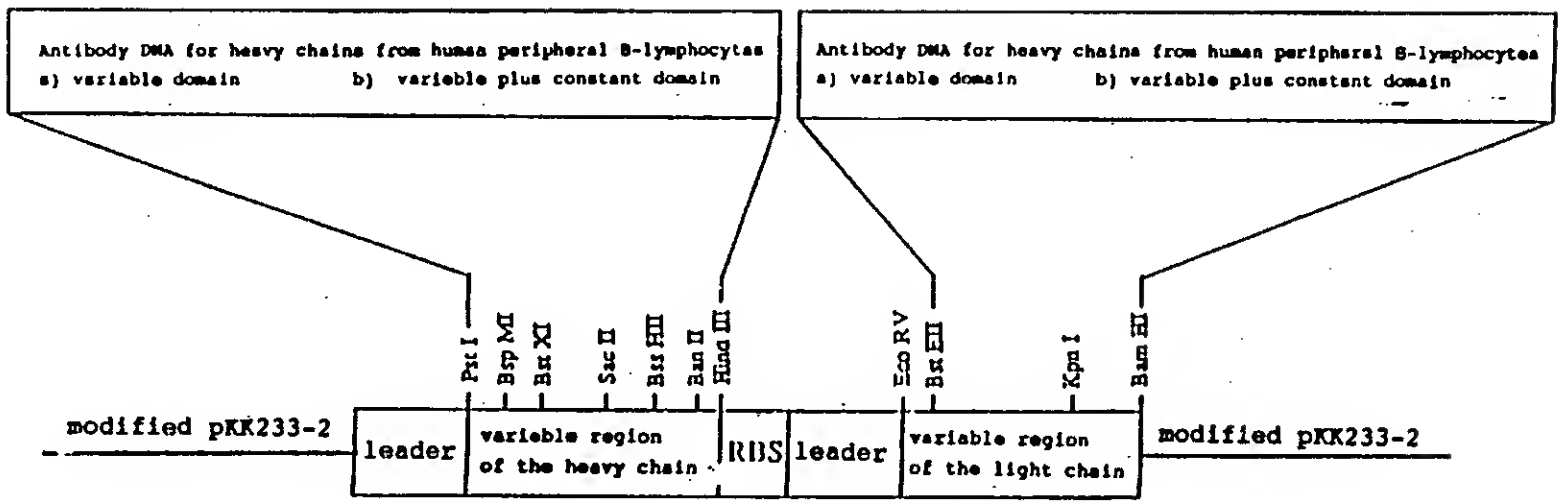
Tab. 6

The Antibody Expression Plasmid pFMT



There is an RBS in the plasmid upstream of the heavy chain part but it is not drawn in here.

TAB. 7 Insertion of the antibody libraries in the expression vector pFMT



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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A human-antibody library obtainable by means of transcription of isolated mRNA from peripheral human B-lymphocytes into cDNA, subsequent amplification of cDNA coding for antibodies by polymerase chain reaction (PCR) by means of suitable primers, and subsequent incorporation into suitable expression plasmids and finally expression, in individual clones, of the relevant antibody RNA which is contained therein and has been amplified in cDNA.

2. A human-antibody library as claimed in claim 1, wherein the expression takes place in the plasmid pFMT.

3. A human-antibody library as claimed in claim 1 or 2, wherein, by selecting suitable primers, only the variable region or a constant domain plus the variable region is amplified in each case.

4. A human-antibody library as claimed in claim 1, 2 or 3, wherein IgM-specific primers are used in the PCR step.

5. A process for preparing a human-antibody library, which comprises mRNA being isolated from peripheral human B-lymphocytes and being transcribed into cDNA, subsequently amplifying the cDNA coding for antibodies by PCR by means of suitable primers, then carrying out an incorporation into suitable expression plasmids and finally expressing the antibody cDNA in individual clones.

6. The process as claimed in claim 5, wherein the expression takes place in plasmid pFMT.

7. The process as claimed in claim 5 or 6, wherein by selecting suitable primers, only the variable region or a constant domain plus the variable region is amplified in each case.

8. A process for isolating specific human antibodies,

comprising screening human-antibody libraries as claimed in claim 1, 2, 3 or 4 using specific antigens.

9. The use of a human-antibody library as claimed in claim 1, 2, 3 or 4 for isolating specific human antibodies.

10. The antibody expression plasmid pFMT.

11. A human-antibody library obtainable by means of transcription of isolated mRNA as claimed in claim 1 and substantially as described herein.

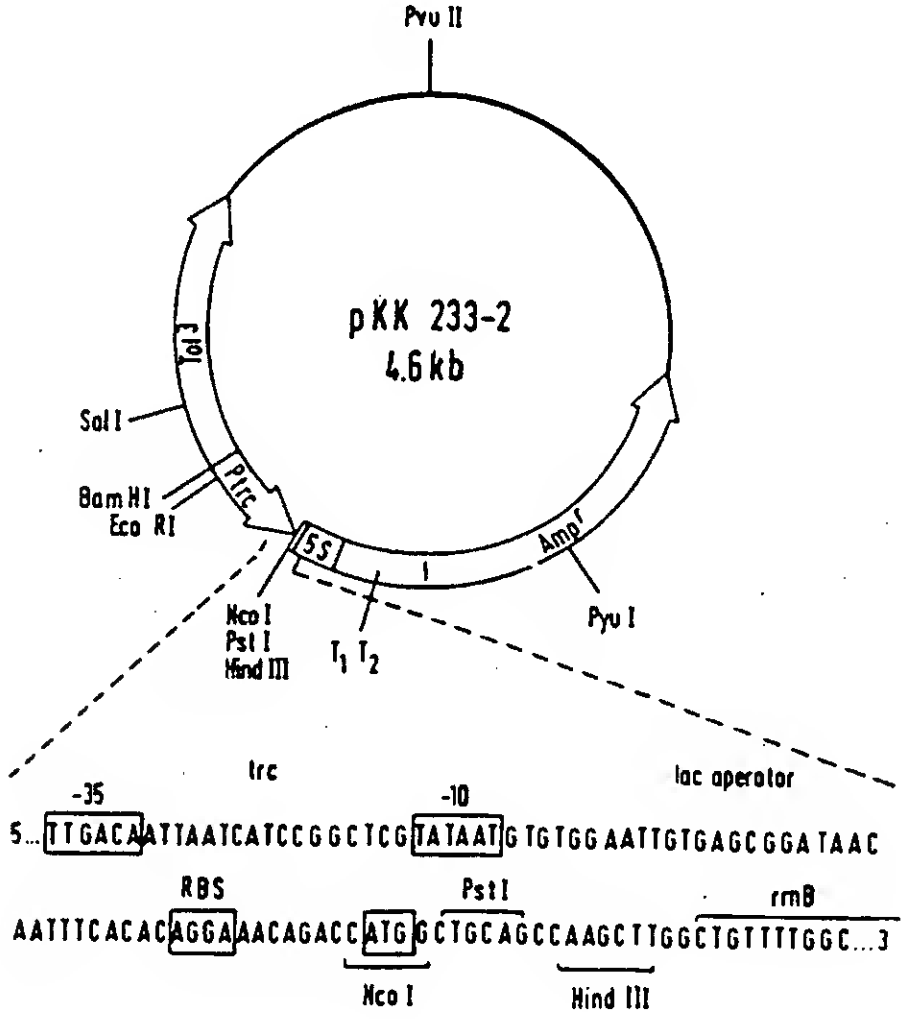

FIG. 1

By: Rogers, Bowden & Penn